Squalene-2,3-Oxide, an Intermediate in the Enzymatic Conversion of Squalene to Lanosterol and Cholesterol*

(Received for publication, May 12, 1967)

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SUMMARY

The conversion of squalene to lanosterol by rat liver preparations in vitro has been re-examined. It has been shown that this transformation, hitherto considered to take place through the agency of one enzyme ("squalene oxidocyclase-1"), actually involves the formation of squalene-2,3-oxide as an intermediate. The oxide is cyclized to lanosterol by a cyclizing system which acts independently of the oxidative step and requires neither oxygen nor NADPH. The cyclase is shown to exist in highest concentration in the microsomal fraction, although an over-all greater quantity of the enzyme remains in the supernatant after centrifugation for 1 hour at 100,000 × g.

Numerous experiments have supported the scheme put forward by Woodward and Bloch for the biological cyclization of squalene to yield lanosterol (1), and the intermediate role of lanosterol in the biosynthesis of cholesterol (2, 3) and ergosterol (4) has been shown. Recent reviews of the relevant literature are available (5, 6). The pattern of folding of the squalene chain proposed by Woodward and Bloch suggested that cyclization was probably initiated by attack of an electrophilic X+ on the double bond at one end of the chain, and this concept has been developed by others (7-9) in schemes which represent the cyclization as a concerted process with the structure of the end-product determined by the conformation of the squalene chain which, in turn, must be the outcome of constraints imposed by the enzyme system that catalyzes the cyclization.

When the cyclization is carried out in the presence of deuterium oxide, no deuterium is found in lanosterol. This result has been interpreted to exclude the formation of partially cyclized intermediates that are stabilized by loss of a proton, since the re-activation of the cyclization of such compounds might be expected to involve the incorporation of a proton from the medium (10).

The rearrangements of methyl groups and hydride ions which must occur in the course of stabilization of the structures of rings C and D of lanosterol has been postulated (8, 10) to take place as a concerted series of 1,2-shifts. The results of several elegant studies which used isotopic labeling methods show that the final outcome of these rearrangements is in complete accord with this hypothesis (11-13).

The exact mechanism of initiation of cyclization has however, remained, until recently, poorly defined. By the use of 18O labeling, Tchen and Bloch (10, 14) showed that the 3β-hydroxyl group of lanosterol was derived from atmospheric oxygen and not from the water of the incubation medium. This result was taken to support the mechanism of initiation of cyclization by OH+ and the view that the enzymatic oxidative and cyclizing activities were probably inseparable was expressed in the naming of the enzyme "squalene oxidocyclase-1." This designation implied that other squalene oxidocyclases must be presumed to be responsible for the conversion of squalene to polycyclic triterpenes other than lanosterol, and in fact the enzymatic conversion of squalene to the pentacyclic triterpene β-amyrin has been shown recently (15). Tchen and Bloch recognized, however, that, on the basis of their results an alternative possibility of proton-initiated cyclization, followed by hydroxylation at C-3, was not entirely excluded, although they reported that attempts to find evidence for it were unsuccessful. Recently some data have been reported which support this possibility (16), but they are unconvincing from the quantitative point of view. The further possibility that enzymatic cyclization of squalene might be initiated by attack of an hydroxyl radical has also been considered (17, 18).

Another possible mechanism, which seemed most attractive to us, involved the initial enzymatic conversion of squalene to its 2,3-oxide (Fig. 1, Route B) and the subsequent initiation of cyclization of this compound by enzymatic electrophilic attack on the oxide ring. Our interest in this possibility arose
from an extended series of studies of the nonenzymatic, acid-catalyzed cyclization of squalene-2,3-oxide and analogous terpenoid oxides whose syntheses are readily achieved by methods developed in one of our laboratories (19). These studies had shown that an electrophilic attack on the oxide ring resulted in fission of the C-2-oxygen bond with cyclization of the carbon skeleton to tricyclic systems. It was noted that the stereochemistry of the products formed in the course of such reactions conformed to that found in rings A and B of the naturally occurring terpenes (Fig. 2) (20–22).

The acid-catalyzed cyclization of analogous oxides has been discussed previously as a formal analogy to the hypothetical OH+-initiated biological cyclization of squalene (23).

If squalene-2,3-oxide is in fact an intermediate in the enzymatic conversion of squalene to lanosterol, it is reasonable to suppose that the step which requires oxygen and NADPH (14) is specifically concerned with the conversion of squalene to its oxide, while lanosterol should be formed from squalene-2,3-oxide anaerobically and without involvement of NADPH. In this paper we describe experiments in which these suppositions have been tested and confirmed. Preliminary accounts of this work have been published (24, 25) and essentially similar results have also been reported in preliminary form by Corey and Russey (26).

**EXPERIMENTAL PROCEDURE**

*Materials.*—L-Mevalonic acid-5-3H (84.5 mC per mmole) and L-mevalonic acid-2-3H (5 mC per mmole) were obtained in the form of the dibenzyl ethylenediamine salts from New England Nuclear and were used without further purification. ATP, NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Mann. Squalene was obtained from Eastman-Kodak. The squalene was purified before use by chromatography on silica gel followed by thiourea clathrate formation (27). The product gave only one peak on gas-liquid chromatography on a column of polydiethylene glycol succinate. This material was used for all preparative work. Lanosterol was purchased from Aldrich and was found by gas liquid chromatography to consist of a mixture of 60% lanosterol and 40% dihydrolanosterol. Pure lanosterol (m.p. 140–141°) was obtained by precipitation of the acetate dibromide and debromination and saponification, essentially as described by Johnston, Gautschi, and Bloch (28). Dihydrolanosterol (m.p. 146–148°) was prepared from purified lanosterol by hydrogenation (29). Cholesterol was obtained from Aldrich and was purified before use by bromination and debromination as described by Fieser (30).

**Gas-Liquid Chromatography.**—Gas-liquid chromatography was carried out by means of a Perkin-Elmer 581 gas chromatograph with all glass columns (6 feet × 1/4 inch inner diameter) and injection systems, with a flame ionization detector and equipped with an effluent stream splitter which permitted collection from four-fifths of the effluent, while the remaining one-fifth was passed to the detector. Materials corresponding to the emergent peaks were collected by inserting glass capillaries of approximately 1-mm bore through a Teflon gasket attached to the outlet of the gas chromatograph. The efficiency of collection by this technique was 30 to 35% of the injected sample. Two types of polar liquid phases were used: temperature stabilized polyethylene glycol succinate obtained as a 5% coating on silanized Chromosorb W, 100 to 120 mesh (Applied Science Laboratories, Inc., State College, Pennsylvania), and a termi- nated Carbowax (“Steroid analytical phase,” Wilkins Instrument Company, Inc., Walnut Creek, California), which was applied to acid-washed, silanized Chromosorb W at 5% concentration. Columns of polyethylene glycol succinate were conditioned at 205° for 48 hours, then operated routinely at 220°, with inlet and outlet temperatures of 300° and a nitrogen flow rate of 90 ml per min. Columns of Carbowax were conditioned first at 250° for 2 hours, then at 230° for a further 15 hours, then operated at 225° with a nitrogen flow rate of 120 ml per min and inlet and outlet temperatures of 300°.

Trimethyl silyl ethers were prepared by treatment of the hydroxyl derivative (<1.0 mg) for 2 hours at room temperature with 0.2 ml of trimethyl silyl chloride and 0.5 ml of anhydrous pyridine. The reagents were then evaporated to dryness under a stream of nitrogen and the products extracted from the residue with hexane. The hexane solution was filtered through a small amount of a mixture of sodium sulfate and anhydrous sodium carbonate, evaporated to dryness and the residue taken up in a few microliters of dry hexane for injection into the gas chromatograph. As a reference compound, cholesterol was added to the injected solution. Retention times are quoted in relation to that of cholestane as Ret values.

For the regeneration of free hydroxylic materials from their
trimethyl silyl ethers after recovery from gas-liquid chromatography, the ether was dissolved in a small volume of a mixture of 70 ml of 95% ethanol and 25 ml of 0.1 N aqueous hydrochloric acid. After 1 hour, the solution was diluted with water and the free hydroxyl group was extracted with hexane.

Some analyses were carried out by conversion of the sterols to methyl ethers and separation on polydiethylene glycol succinate as previously described (31).

Radioactivity Measurements—A Packard liquid scintillation counter operating with an efficiency of 41% for $^3$H and 89% for $^14$C was used. Samples were assayed in 20-ml glass vials in scintillation fluid containing 2,5-diphenyloxazole W was used. Samples were assayed in 20-ml glass vials in scintillation fluid containing 2,5-diphenyloxazole (5 g) and 1,4-bis-2-(5-phenyloxazolyl)benzene (0.3 g) per liter of toluene. All radioactivity values reported are reliable to better than ±5%.

Mass Spectrometry—Mass spectrometry was carried out by Dr. Heinrich Schnoes in the laboratory of Dr. A. L. Burlingame with the use of a CEC mass spectograph.

Infrared Spectra—These spectra were measured in chloroform solution by means of a Perkin-Elmer 257 spectrophotometer.

Nuclear Magnetic Resonance Spectra—A Varian A60 nuclear magnetic resonance spectrometer with tetramethyl silane as an internal reference was used.

Thin Layer Chromatography—Thin layer chromatography was carried out on glass plates coated with 0.25-mm layers of Silica Gel G (10% calcium sulfate binder). The following solvent systems were used: 5% ethyl acetate in hexane (System I) when the separation of squalene from squalene oxide was the main requirement, 25% ethyl acetate in hexane (System II) for the isolation of lanosterol, or 15% ethyl acetate in hexane (System III) for some other preparative purposes. Radioactive incubation products were localized on the plates by chromatographing known reference substances near the edges of the plate and visualizing these by spraying with a solution of iodine in hexane while covering the central portion of the chromatogram with a glass plate. The bands corresponding to the reference substances were then scraped from the plate and extracted with a suitable solvent. A mixture of methylene chloride and methanol (1:1) was used for extraction of all materials other than squalene oxide for which methylene chloride was used alone to avoid the possibility of hydrolytic attack on the oxide ring. Extractions were carried out by suspending the silica gel in the solvent in a centrifuge tube followed by evaporation of the solvent after centrifugation.

Squalene-2,3-oxide—Squalene-2,3-oxide was prepared by the following procedure, based on the earlier procedure of van Tamelen and Curphey (19).

N-Bromosuccinimide, 12.5 g (0.07 mole), was added in small portions over a period of 10 min to a solution of 25 g (0.06 mole) of squalene in 430 ml of tetrahydrofuran and 120 ml of water. The additions were made under an atmosphere of nitrogen which was maintained while the solution was stirred at 5°C for a further 60 min. After evaporation of the major portion of the solvent under reduced pressure the residual aqueous mixture was poured into ice water and, extracted with benzene, and the combined benzene extracts were dried over sodium sulfate to yield 29.2 g of crude monobromohyrin. The crude product was dissolved in petroleum ether (b.p. 40–45°C) and applied to a column of silica gel (800 g, 15% water) made up in the same solvent. Unchanged squalene and nonhydroxyllic bromination products were eluted with petroleum ether. The bromohydrin (11.8 g) was eluted with 20 to 50% benzene in petroleum ether.

The foregoing bromohydrin was dissolved in 250 ml of methanol under nitrogen and 13.8 g of anhydrous potassium carbonate were added in one portion. The mixture was stirred for 1 hour at 25°C, evaporated to dryness under reduced pressure, and the residue was extracted exhaustively with ether. The combined ether extracts were washed with water, dried over sodium sulfate, and evaporated under reduced pressure to yield 6.3 g of squalene oxide of better than 95% purity. The purity of small amounts of the oxide was routinely determined by thin layer chromatography in System I in which the oxide moves with an $R_F$ of 0.41 to 0.43.

The following analytical data support the structural assignment.

\[
\begin{align*}
C_{30}H_{50}O \\
\text{Calculated:} & \quad C 84.18, H 11.78 \\
\text{Found:} & \quad C 84.44, H 11.81 \\
\end{align*}
\]

Mass spectrum (cf. Fig. 3a) showed a molecular ion peak at $m/e = 426$, with major fragments of $m/e = 357, 203, 191, 189, 177, 175, 163, 161, 153, 149, 147, 137, 123, 121, 109, 107, 105, 93, 79, 77, 65, 53$, and 137 cm$^{-1}$. Nuclear magnetic resonance spectral data for squalene-2,3-oxide are given in Table I.

\[
\begin{align*}
\text{C}_9\text{H}_{16}\text{O} \\
\text{Calculated:} & \quad C 84.18, H 12.23 \\
\text{Found:} & \quad C 83.98, H 12.02 \\
\end{align*}
\]
On mass spectrometry, a molecular ion peak was obtained at $m/e = 429$ with major fragments at $m/e = 410, 341, 290, 279, 263, 223, 191, 178, 163$, and $149$. The infrared spectrum maxima were $3630, 2940, 2865, 1660$ (weak), $1524$ (weak), $1445, 1375, 1245$, and $855$ cm$^{-1}$. Nuclear magnetic resonance spectral data for 2,3-dihydro-2-hydroxysqualene are given in Table II.

Squalene-2,3-glycol and 1,1',2-Trisnorsqualene-3-aldehyde—
The preparation of these compounds on a large scale, by methods that are essentially similar to the procedures described (see “Results”) for their formation from submilligram quantities of biosynthetically labeled squalene-2,3-oxide, will be described in detail elsewhere. The authenticity of these compounds is based on the following analytical data.

The elemental analysis of squalene-2,3-glycol gave

$$C_{30}H_{44}O_2$$

Calculated: C 81.02, H 11.79
Found: C 81.42, H 11.32

On mass spectrometry the molecular ion, $m/e = 444$, was most abundant, with major fragments of $m/e = 429, 426, 385, 375, 367, 357, 289, 231, 149, 143, 137, 123, 109, 95, 81, 69, 59$, and $55$. The infra-red spectrum gave maxima at $3580, 2965, 2929, 2845, 1665, 1450, 1380$, and $1060$ cm$^{-1}$. The nuclear magnetic resonance spectrum for squalene-2,3-glycol is given in Table III.

The elemental analysis of 1,1',2-trisnorsqualene-3-aldehyde gave

$$C_{30}H_{40}O$$

Calculated: C 84.31, H 11.53
Found: C 84.35, H 11.44

On mass spectrometry the molecular ion, $m/e = 384$, was most abundant, with major fragments of $m/e = 369, 356, 341, 315, 273, 209, 205, 192, 161, 136, 111, 93, 81, 69$, and $55$. The infra-red spectrum was essentially similar to that of squalene with additional bands at $3200$ (W) (aldehydic hydrogen stretching) and $1720$ cm$^{-1}$ (carbonyl). Nuclear magnetic resonance spectral data for 1,1',2-trisnorsqualene-3-aldehyde are given in Table IV.

Preparation of Homogenates of Rat Liver—Homogenates of the livers of male rats (100 to 150 g) were prepared essentially according to the method of Bucher and McGarrahan (32). All operations were carried out at 2–4°C and all equipment was precooled in ice. In a typical preparation, the livers of 10 rats were chopped with a razor blade and ground for ½ min, using a Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle.

The homogenization was carried out in small batches (2 livers per run) with 2.5 ml of buffered medium per gram of tissue. The particulate fractions were washed twice by rehomogenization in a volume of buffer equal to one-half of the total or original volume of homogenate and 5- and 50-μl aliquots of each fraction were taken for protein assay by the method of Lowry et al. (33).

Incubations—Incubations were carried out for 2 hours in a Dubnoff shaking incubator at 37°C in stopped Erlenmeyer flasks containing appropriate volumes of homogenate or subcellular fractions and flushed for 2 min with either oxygen or...
Squalene-2,3-oxide in Sterol Biosynthesis

RESULTS

Biosynthesis of Squalene-2,3-oxide-\(^3\)H—Squalene (20 \(\mu\)g, 47,100 dpm per \(\mu\)g), biosynthetically labeled from mevalonic acid-5-\(^3\)H, was incubated with 3 ml of the \(S_1\) supernatant of rat liver homogenate in the presence of the NADPH-generating system under an atmosphere of oxygen. Duplicate incubations were carried out with and without the addition of 300 \(\mu\)g of unlabeled squalene oxide. The products were isolated by saponification and extraction with hexane, followed by thin layer chromatography in System I. Squalene-2,3-oxide (\(R_F = 0.41\)) was clearly separated from squalene (\(R_F = 0.8\)), lanosterol (\(R_F = 0.1\)), and cholesterol (\(R_F = 0.045\)). The bands corresponding to squalene, squalene oxide, and total sterols were removed from the plate, extracted, and assayed for radioactivity. Recoveries of 90% of the total initial radioactivity were achieved. The distribution of recovered activity was, in the experiments in which 300 \(\mu\)g of unlabeled squalene oxide were present, as follows: squalene, 69.5%; sterols, 11.1%; squalene oxide, 7.2%. In the incubations from which squalene oxide was omitted, there was a markedly lower incorporation of squalene into the oxide (2.2%) and an enhanced incorporation into the combined sterol fractions (19.3%) while the recovery of unchanged squalene was 68.5%.

Conversion of Squalene-2,3-oxide-\(^3\)H to Squalene-2,3-glycol-\(^3\)H—Squalene-2,3-oxide containing 40,900 dpm that had been isolated from the foregoing incubations in the presence of 300 \(\mu\)g of unlabeled squalene oxide was dissolved in 500 \(\mu\)l of tetrahydrofuran and water (7:3), 250 \(\mu\)l of 3% perchloric acid were added under nitrogen, and the mixture was allowed to stand at 4°C for 12 hours. The tetrahydrofuran was evaporated under nitrogen and the product was extracted with six 1-ml portions of cyclohexane. The combined extracts were dried over anhydrous sodium sulfate and concentrated in a stream of nitrogen to yield material with a total of 35,968 dpm.

A sample of this material (31,000 dpm) was subjected to thin layer chromatography in System II, giving a band corresponding to squalene-2,3-oxide. The radioactivity of the band was assayed, and the product was extracted with three 1-ml portions of cyclohexane. The combined extracts were dried over anhydrous sodium sulfate and concentrated in a stream of nitrogen to yield material with a total of 18,380 dpm.

The mass spectrum of this material in the region of the molecular ions is shown in Fig. 3b. From measurements of the intensities of the peaks corresponding to \(m/e = 426\) (\(^3\)O-oxide, molecular ion) and \(m/e = 428\) (\(^1\)O-oxide, molecular ion) it was determined that the product of the above reaction contained \(^1\)O in the same abundance (30%) as the water used in the reaction.

FIG. 3. Portions of the mass spectra in the region of the molecular ion peaks, of A, normal squalene-2,3-oxide; B, squalene-2,3-oxide-\(^3\)O; C, normal lanosterol; and D, lanosterol formed enzymatically from 13.

Squalene-2,3,5-oxide-\(^3\)H—This was prepared by a small scale modification of the procedure described for the preparation of unlabeled material.

Biologically labeled squalene-\(^3\)H (6.1 mg, 4350 dpm per \(\mu\)g), treated with 2.64 mg of \(N\)-bromosuccinimide in a mixture of 0.15 ml of tetrahydrofuran and 0.05 ml of water (30% \(^1\)O), and worked up as described above, gave a bromohydrin which was isolated by thin layer chromatography in Solvent System III, with an \(R_F\) of 0.4. Treatment of this material with anhydrous potassium carbonate in methanol and separation of the product by thin layer chromatography in System I gave 1.16 mg of squalene-2,3-oxide-\(^3\)H-\(^1\)O (\(R_F = 0.41\)), specific activity, 4150 dpm per \(\mu\)g.

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to the glycol ($R_F = 0.21$) from which material having 27,000 dpm was recovered. This represents a conversion of 77% of the starting material to the glycol.

**Cleavage of Squalene-2,3-glycol-3H to 1,1',2-Trisnorlanosterol-3-aldehyde-3H**—A sample of the isolated glycol containing 19,000 dpm was dissolved in 1 ml of tetrahydrofuran. Excess sodium metaperiodate was added, followed by sufficient water to make a homogeneous solution which was allowed to stand at 4°C under nitrogen for 16 hours. The tetrahydrofuran was removed in a stream of nitrogen, and the product extracted in cyclohexane. The extracts were combined, dried, and evaporated in a stream of nitrogen, to yield a crude aldehyde fraction with 15,350 dpm. The bulk of the above product (containing 15,000 dpm) was purified by thin layer chromatography in System I and the band corresponding to the aldehyde ($R_F = 0.17$) was eluted. It contained 11,600 dpm (77%). A portion of the aldehyde (9,400 dpm) was subjected to gas-liquid chromatography on a column of Carbowax with effluent collection during 2-min intervals. The collected fractions were assayed for radioactivity and only two radioactive peaks were detected. Peak I ($R_F = 2.7$) corresponded to 24,25-dihydroxylanosterol methyl silyl ether and yielded 7180 dpm of $^{14}$C; Peak II ($R_F = 3.7$) corresponded to lanosterol methyl silyl ether and yielded 3850 dpm of $^{14}$C. There was negligible radioactivity in other parts of the chromatogram. In four subsequent experiments the ratios of the areas and associated radioactivity of the lanosterol and dihydroxylanosterol peaks varied from approximately 2:1 to 1:2 as in the present experiment.

**Further Identification of Materials of Peaks I and II**—From a similar experiment to the above, in which squalene-2,3-oxide-3H (6000 dpm per $\mu$g) was incubated with the $S_1$ supernatant containing a NADPH-generating system, materials corresponding to Peaks I and II were isolated.

Material corresponding to Peak I (17,350 dpm) was combined and crystallized with 20 mg of unlabeled dihydrolanosterol giving 18 mg of material with 800 dpm per mg, which was unchanged during four recrystallizations from methanol and methylene chloride. Acetylation at room temperature with acetic anhydride and pyridine, followed by four further crystallizations from acetone, gave dihydrolanosteryl acetate, m.p. 120–121°C, the specific activity of which remained constant at 730 dpm per mg.

A sample of the material of Peak II which contained 35,000 dpm was dissolved in 5% acetic acid in ethyl acetate and hydrogenated at 25°C and at atmospheric pressure in the presence of a platinum catalyst. The product was isolated by evaporation of the solvent, converted to the trimethyl silyl ether, and subjected to gas-liquid chromatography with collection of effluent material. Only one radioactive peak emerged, containing 14,180 dpm. Its retention time ($R_T = 2.7$) corresponded with that of 24,25-dihydroxylanosterol trimethyl silyl ether.

**Identification of Lanosterol as Lanosteryl Acetate 24,25-Dibromide**—The foregoing results are consistent with the enzymatic conversion of squalene-2,3-oxide to a mixture of lanosterol and dihydrolanosterol. Further evidence for the composition of this mixture was obtained as follows. A portion of material, isolated by thin layer chromatography as described above and containing 14,100 dpm $^{3}$H, was combined with 2 mg of pure lanosterol. It was acetylated by overnight treatment with pyridine and acetic anhydride followed by removal of the excess reagents under a stream of nitrogen. Thin layer chromatography of the product in System I gave 1.72 mg of a white solid, $R_T = 0.58$ (corresponding to lanosteryl acetate) containing 11,000 dpm of $^{14}$C. This product was diluted to 25 mg with unlabeled lanosteryl acetate (m.p. 130–131°C) and crystallized to give 21 mg of material with a specific activity of 431 dpm per mg, which remained unchanged during four crystallizations. A portion of the recombined, recrystallized material and mather liquids (19.6 mg) was dissolved in 0.8 ml of acetic acid saturated with potassium.
bromide and treated with 25 μl of 10% bromine in acetic acid (0.043 mmole). After 10 min at room temperature, 30 μl of water were added and the mixture was allowed to stand at 4°C overnight. The crystalline dibromide was collected by centrifugation and washed with cold acetic acid, followed by two portions of cold methanol. The crystals were dried in a stream of nitrogen to yield 10.93 mg of white crystalline material (specific activity, 276 dpm per mg). On recrystallization from acetone, 6.10 mg of pure crystalline dibromide, m.p. 167–169°C were obtained, with a specific activity of 91 dpm per mg, which was unchanged (91 to 96 dpm) on two further crystallizations. These results are consistent with the presence of 28 to 30% of the radioactivity in lanosterol.

**Enzymatic Conversion of Material of Peak II (Lanosterol) to Cholesterol**—Material corresponding to Peak II, having 4600 dpm 14C, was distributed between two 3 ml aliquots of S1 supernatant containing the NADPH-generating system and incubated for 2 hours under oxygen. The nonsaponifiable fraction was isolated in the usual way and subjected to thin layer chromatography on Solvent System II. Of the recovered radioactivity, 1100 dpm (32%) were associated with the cholesterol fraction; 1100 dpm remained associated with lanosterol, and a further 15% was recovered from an intermediate fraction. The radioactive material moving chromatographically with cholesterol and containing 1000 dpm was combined with 20 mg of cholesterol and crystallized to give 18 mg of material having a specific activity of 50 dpm per mg. This material was acetylated with acetic anhydride and pyridine and the product was crystallized from acetic acid to yield 1000 dpm. On recrystallization from acetone, 6.10 mg of pure crystalline dibromide, m.p. 167–169°C were obtained, with a specific activity of 91 dpm per mg, which was unchanged (91 to 96 dpm) on two further crystallizations. These results are consistent with the presence of 28 to 30% of the radioactivity in lanosterol.

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**Gas-Liquid Chromatography** of the methyl ester of the radioactive material (750 μg) of squalene-2,3-oxide-3H, 5000 dpm per pg. The nonsaponifiable fraction was isolated in the usual way and separated by thin layer chromatography in System II. The bands corresponding to squalene-2,3-oxide and lanosterol (the only significantly labeled areas) were assayed for radioactivity, and the conversion of squalene-2,3-oxide to lanosterol was calculated from the amount of radioactivity associated with the lanosterol band, as a percentage of total recovered nonsaponifiable material. Control incubations carried out with boiled enzyme preparations showed that the amount of activity of squalene-2,3-oxide which appeared in the lanosterol region on thin layer chromatography as a result of nonenzymatic transformation was less than 5% of the lowest enzymatic conversion, and therefore no correction was applied. The protein contents of individual fractions were assayed, and the results are reported in Table V as specific activities (micrograms of lanosterol formed per mg of protein) and the percentage of total recovered cyclizing activity per fraction.

**Enzymatic Conversion of Squalene-2,3-oxide-H3O to Lanosterol-H2O**—A sample (750 μg) of squalene-2,3-oxide-H3O, 4150 dpm per μg, 30% 3H0, was incubated anaerobically for 2 hours with a total of 60 ml of washed microsomal fractions without addition of the NADPH-generating system. Isolation of the nonsaponifiable fraction in the usual way gave 7.62 mg of material containing 2.4 × 104 dpm. Thin layer chromatography of this product in Solvent System II gave unambiguous evidence of squalene-2,3-oxide, cholesterol, and lanosterol. The area of the chromatogram corresponding to Δ5-cholesterol methyl ether (Rf = 4.7). When a sample (200 μg) of the labeled cholesterol acetate, which had been purified by the dibromide, was converted to the methyl ether and analyzed by gas-liquid chromatography under similar conditions, all recovered radioactivity was associated with the cholesterol methyl ether peak (Rf = 3.85). In this case the effluent materials corresponding to the peak were collected as three consecutive fractions which showed relative specific activities of 5.2, 5.0, and 5.3 dpm per cm2, respectively.

In a similar experiment reported previously (24), no loss of specific activity was detectable during purification of the cholesterol acetate as the dibromide, and when the material was converted to the methyl ether and analyzed by gas-liquid chromatography on polyethylene succinate, the peak corresponding to cholesterol methyl ether contained all of the radioactivity that was associated with cholesterol on thin layer chromatography.
Distribution of 2,3-oxido-squalene cyclase activity in fractions of rat liver homogenates

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Oxide cyclized&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Distribution of recovered cyclizing activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.30</td>
<td>(100)</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>0.15</td>
<td>9.4</td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>0.30</td>
<td>9.4</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>1.10</td>
<td>37.5</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.30</td>
<td>44.0</td>
</tr>
<tr>
<td>Nuclear plus supernatant&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Mitochondria plus supernatant&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Microsomes plus supernatant&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Microsomal fraction plus NADPH-generating system</td>
<td>1.10</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> For conditions see “Experimental Procedure.” Average of two experiments in duplicate.

<sup>b</sup> Values for individual fractions calculated as the percentage of total recovery. Total recovery of activity in homogenate fractions was actually 105 to 110% of activity in the unfractionated homogenate. The significance of this result is not clear at the present time.

<sup>c</sup> Each fraction (1.5 ml) was prepared as under “Experimental Procedure.”

was collected. The products recovered from one run with collection of fractions during 1-min intervals were assayed for radioactivity. Two peaks of material emerged. One (R<sub>m</sub> = 1.6) contained no radioactivity, while the other (R<sub>m</sub> = 3.7, identical with lanosterol trimethyl silyl ether) was associated with all of the recovered radioactivity. The combined recovered materials corresponding to this peak obtained from the following three runs were analyzed by mass spectrometry. The mass spectrum is shown in Fig. 2d and may be compared with that of normal lanosterol (Fig. 2c). Calculation of the abundance of <sup>18</sup>O from the relative intensities of the peaks corresponding to the molecular ions with m/e = 498 (<sup>18</sup>O-lanosterol-trimethyl silyl ether) and m/e = 500 (<sup>18</sup>O-lanosterol-trimethyl silyl ether) shows the presence of 29% <sup>18</sup>O in the recovered material.

DISCUSSION

The experiments described in this paper were designed to explore the possibility that the conversion of squalene to lanosterol, hitherto considered to take place as the result of the activity of a single enzyme, “squalene oxidocyclase-1” (14) according to Route A (Fig. 1), actually entailed at least two steps. These are depicted as Route B (Fig. 1) and involved (a) the oxidation of squalene to its 2,3-oxide and (b) the cyclization of this compound by an acid-initiated process to give lanosterol. It is implicit in the proposed mechanism of the cyclization step that the oxygen of the 2,3-oxide ring should become incorporated into the lanosterol molecule in the form of the 3β-hydroxy group. All of the results reported in this paper are in accord with the sequential oxidation and cyclization mechanism.

When an homogenate of rat liver was incubated with radioactive squalene and an exogenous pool of unlabeled squalene-2,3-oxide, radioactivity accumulated in squalene-2,3-oxide and the incorporation of radioactivity into the sterols was correspondingly reduced, in comparison with control incubations to which no unlabeled oxide was added. The conclusion that the accumulated radioactivity resides in squalene-2,3-oxide is based on the following observations. The radioactivity moves with authentic squalene oxide on thin layer chromatography in 5% ethyl acetate in hexane, a solvent system which clearly separates squalene-2,3-oxide from squalene as well as from isomeric oxides of squalene and squalene-2,3- and 22,23-dioxide. When the labeled 2,3-oxide was hydrolyzed under aqueous acidic conditions, a single product was obtained, which had the mobility on thin layer chromatography of 2,3-dihydro-2,3-dihydroxy squalene and showed no loss of radioactivity. When this material was oxidized with periodate, a new radioactive derivative was obtained which had the mobility on both thin layer chromatography and gas-liquid chromatography of authentic 1,1',2-trimersqualene aldehyde and had a specific activity close to that of the oxide. On reduction with lithium aluminum hydride, the labeled 2,3-oxide gave a single product which both on thin layer and gas-liquid chromatography (as the trimethyl silyl ether) had the mobility of 2,3-dihydro-2-hydroxy squalene and retained the original specific activity.

The various derivatives utilized in the above characterization procedures have been synthesized independently in our laboratory and fully characterized. All analytical data available are consistent with the postulated structures, and the identity of the biologically labeled material with squalene-2,3-oxide is strongly supported by the retention of labeling through the reactions described. Its conversion to cholesterol under aerobic incubation conditions gives further evidence for the role of squalene-2,3-oxide as a normal intermediate in the enzymatic conversion of squalene to sterols.

The first 3β-hydroxy sterol to arise from the cyclization of squalene 2,3 oxide, according to Route B, Fig. 1, would be expected to be lanosterol; but, on anaerobic incubation in the presence of a NADPH-generating system and the S<sub>1</sub> supernatant of the homogenate, there should also be an appreciable conversion of lanosterol to dihydrolanosterol, mediated by a microsomal NADPH-dependent Δ<sup>2</sup> reductase (34). Lanosterol and dihydrolanosterol were identified as products of the incubation of labeled squalene oxide with the S<sub>1</sub> supernatant, first on the basis of their mobilities on thin layer chromatography as the free sterols and the acetates, and on gas-liquid chromatography as the trimethyl silyl ethers. The radioactive material associated with the peak of lanosterol trimethyl silyl ether, on gas-liquid chromatography, was further identified by hydrogenation to a product which, as the trimethyl silyl ether, had a retention time identical with that of dihydrolanosterol. Radioactive material isolated from the original incubation mixture and having the retention time of dihydrolanosterol on gas-liquid chromatography (as the trimethyl silyl ether) showed no loss of radioactivity when recrystallized with authentic dihydrolanosterol either as the free sterol or after acetylation.

Furthermore, when a portion of the labeled material which behaved like lanosterol on thin layer chromatography was combined with pure lanosterol, acetylated, and recrystallized, no loss of radioactivity was detected. When this mixture was brominated, however, the resulting lanosterol-3β-acetate-24,25-dibromide lost more than two-thirds of its radioactivity. These

<sup>2</sup> It has so far proved impossible to obtain satisfactory results in attempts to analyze squalene-2,3-oxide and closely related analogues by gas-liquid chromatography, owing to the formation of thermal degradation products.
results are consistent with the presence of the radioactivity of the material isolated by thin layer chromatography, in a mixture of lanosterol and dihydroxolanosterol, since it is well known that the acetates of these substances are extremely difficult to separate by crystallization (35), but are readily separable on the basis of the reactivity of the $\Delta^2$-bond to bromine (28). The further metabolism of the lanosterol formed in these experiments to olefin under aerobic conditions gives additional support for authenticity of the cyclization product.

A direct demonstration of the metabolic conversion of squalene-2,3-oxide to lanosterol as the sole product, under nonreducing conditions, was achieved in the experiment involving the anaerobic incubation of squalene-2,3-oxide-18O with washed microsomes. A yield of lanosterol of approximately 7% was obtained from 750 $\mu$g of squalene oxide, and the isolation of the lanosterol by gas-liquid chromatography of the trimethylsilyl ether showed no detectable quantity of the dihydro compound. The quantitative retention of 18O in the conversion of squalene-oxide-18O-1H to lanosterol-18O-1H is evident from the comparison (Fig. 3) of the regions of the mass spectra in the vicinity of the molecular ions of (a) normal squalene 2,3-oxide and (b) squalene 2,3-oxide labeled with 18O, and (c) of normal lanosterol, and (d) lanosterol isolated from the incubation of squalene-2,3-oxide-18O. The abundance of the molecular ion of m/e 426 (squalene-2,3-oxide-18O) is 30%, as shown in Fig. 3b, and the molecular ion of m/e 300 (lanosterol trimethylsilyl ether-18O) appears in the mass spectrum of the lanosterol biologically derived from the 18O-labeled oxide, with an abundance of 28%. These results further substantiate the proposed mechanism of enzymatic cyclization of squalene-2,3-oxide according to Fig. 1, Route B.

It has been shown by Tchen and Bloch (14) that the complete enzyme system for the conversion of squalene to lanosterol is fully active only when the microsomal and supernatant fractions are combined, and that oxygen and NADPH are required for this conversion. In the course of our experiments we have obtained similar results. Since our major current interest is in the characteristics of the cyclizing system, we have carried out a preliminary study of its distribution, the results of which are shown in Table V.

It is clear that the greatest concentration of cyclase activity per mg of protein is in the microsomal fraction. Calculation of the over-all distribution of total cyclizing activity, however, indicates that a large portion of it (though at a lower specific activity) is present in the cell sap. It seems probable that, at least in part, this reflects a failure of some small microsomal fragments to sediment under our conditions of centrifugation. The alternative possibilities, that some degree of solubilization of the enzyme occurs during homogenization or that there is a portion of the cyclizing activity which is normally present in a soluble state, encourages us to believe that true solubilization of the enzyme may be accomplished.

It is evident from the results shown in Table V that the cyclizing enzyme system is equally effective with or without the addition of the NADPH-generating system. The lack of a requirement both for NADPH and for oxygen is consistent with the concept that the cyclization is an acid-initiated process that does not involve either oxidative or reductive steps. The results are in accord with the expectation that the dependence on these cofactors, originally attributed to the "squalene oxidocyclase" system (14), must actually reflect the requirements of the oxidase system. The characteristics of this enzyme system have not so far been established in our laboratory, but are under examination at the present time.

The results reported here strongly support the proposed role of squalene-2,3-oxide as a naturally occurring intermediate in the biological conversion of squalene to lanosterol. It seems highly probable that the oxide plays a similar role in the biogenesis of the extensive array of tetr- and pentacyclic triterpenes hitherto considered as cyclization products of squalene. Some aspects of the analogy of these biological cyclizations to nonenzymatic cyclizations of squalene-2,3-oxide have been noted elsewhere (37).

It is clear that the identification of the 2,3-oxide of squalene as an intermediate in the cyclization of squalene allows a wide range of new approaches to the problem of the mechanism of cyclization. Not only is the enzyme that catalyzes the cyclization a simpler and more tractable entity than "squalene oxidocyclase-I" was believed to be, but the possibility of dealing with a substrate (the oxide) which is asymmetrical, rather than with squalene itself, which is symmetrical, facilitates the synthesis of a variety of analogues which can be used to test specific concepts concerning the mechanism of action of the cyclase. Current studies in our laboratories are being directed along these lines.

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